Basic Science
Research Paper

OPEN

PAIN REPORTS®

Effects of inflammatory pain on CB1 receptor in the midbrain periaqueductal gray

Adrianne R. Wilson-Poe[a,*], Beth Wiese[b], Cherkaouia Kibaly[c], Lindsay Lueptow[c], Jeniffer Garcia[b], Preeti Anand[b], Catherine Cahill[c], Jose A. Morón[b]

Abstract

Introduction: The periaqueductal gray (PAG) mediates the antinociceptive properties of analgesics, including opioids and cannabinoids. Administration of either opioids or cannabinoids into the PAG induces antinociception. However, most studies characterizing the antinociceptive properties of cannabinoids in the PAG have been conducted in naive animals. Few studies have reported on the role of CB1 receptors in the PAG during conditions which would prompt the administration of analgesics, namely, during pain states.

Objectives: To examine inflammatory pain-induced changes in CB1 receptor expression and function in the midbrain periaqueductal gray.

Methods: In this study, we used the Complete Freund Adjuvant model to characterize CB1 receptor expression and G-protein coupling during persistent inflammatory pain.

Results: Inflammatory pain induced an upregulation in the expression of synaptic CB1 receptors in the PAG. Despite this pain-induced change in CB1 expression, there was no corresponding upregulation of CB1 mRNA after the induction of inflammatory pain, suggesting a pain-induced recruitment of CB1 receptors to the synaptic sites within PAG neurons or increased coupling efficiency between the receptor and effector systems. Inflammatory pain also enhanced ventrolateral PAG CB1 receptor activity, as there was an increase in CP55,940-stimulated G-protein activation compared with pain-naïve control animals.

Conclusion: These findings complement a growing body of evidence which demonstrate pain-induced changes in brain regions that are responsible for both the analgesic and rewarding properties of analgesic pharmacotherapies. Because much of our understanding of the pharmacology of cannabinoids is based on studies which use largely pain-naïve male animals, this work fills in important gaps in the knowledge base by incorporating pain-induced adaptations and cannabinoid pharmacology in females.

Keywords: Cannabinoid, CB1, Inflammatory pain, Periaqueductal gray, PAG

1. Introduction

The rapidly changing landscape of cannabis legislation in the United States has led to a large number of patients using cannabis to manage chronic pain. Although the analgesic properties of delta-9-tetrahydrocannabinol (THC), the main psychoactive ingredient in cannabis, are well documented, most preclinical cannabinoid research has been conducted by studying these compounds in pain-naïve animals. A rapidly growing body of evidence suggests that pain itself creates long-lasting adaptations in brain circuitry that are responsible for somatic and affective pain modulation, as well as the rewarding properties of analgesic drugs.[7,13,15,26] Thus, it is imperative to characterize the therapeutic risks and benefits of cannabis and cannabinoids such as THC in validated preclinical models of persistent and chronic pain. Although our previous studies have examined the impact of pain on opioid receptor expression and function, persistent pain-induced changes in the cannabinoid systems are not as well characterized.

The midbrain periaqueductal gray (PAG) is critically involved in descending pain modulation, and it mediates the antinociceptive properties of both opioids and cannabinoids. Administration of either opioids or cannabinoids directly into the PAG induces antinociception.[11,22,39] The presumed analgesic mechanism of cannabinoids is the modulation of presynaptic glutamate and GABA release, given that CB1 agonists reduce both excitatory and inhibitory postsynaptic currents in the PAG.[40] Indeed, knocking out CB1 receptors on either glutamatergic or GABAergic neurons in the PAG abolishes electroacupuncture-induced antinociception.[50] Such as opioids, cannabinoids modulate the probability of neurotransmitter release, but unlike opioids, do not have a direct postsynaptic effect on PAG neurons.[40]

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

[a] *Department of Neurobiology, Legacy Research Institute, Portland, OR, USA,* [b] *Departments of Anesthesiology, Neuroscience and Psychiatry, Washington University in St. Louis, St. Louis, MO, USA,* [c] *Department of Psychiatry and Biobehavioral Sciences, University of California, Los Angeles, CA, USA*

**Corresponding author. Address: Department of Neurobiology, Legacy Research Institute, Portland, OR 97232, USA. Tel.: +1 (503) 413-1754. E-mail address: arae@downeurobiology.org (A.R. Wilson-Poe).*

PR9 6 (2021) e897

http://dx.doi.org/10.1097/PR9.0000000000000897

The PAG also plays an important role in depression-like behavior.[12,16,19,42] Depression and chronic pain are frequently comorbid; up to 85% of chronic pain patients experience depression.[2] Indeed, our recent work supports the neurobiological link between the affective and somatic components of pain.[8,28] The CB1 receptor may be implicated in this link, given that CB1 regulates the affective component of pain in cortical regions, including those the anterior cingulate cortex which projects to the PAG.[3,33]

Despite the critical role that the PAG seems to play in both the affective and somatic components of pain, most studies characterizing the antinociceptive properties of cannabinoids in the PAG have been conducted in pain-naive animals. Few studies have reported on the role of CB1 receptors in the PAG during conditions which would prompt the administration of analgesics, namely, in persistent or chronic pain. Because chronic pain induces long-lasting adaptations in the central nervous system, it is imperative to understand how these adaptations may affect the analgesia that is produced by cannabinoids, alone or in combination with opioids.

2. Methods

2.1. Animals and Complete Freund Adjuvant–induced inflammatory pain

All experiments were approved by the Institutional Care and Use Committees of Washington University in St. Louis and Washington State University, in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Male and female Long Evans rats (60–90 days) were pair housed with a 12/12 hours dark/light cycle and acclimated to the vivarium for at least 7 d before any manipulation. Rats received food ad libitum throughout the behavioral studies. We used the Complete Freund Adjuvant (CFA) model of inflammatory pain. Complete Freund Adjuvant (Calbiochem) was diluted in the same volume of sterile saline before its unilateral subcutaneous injection of 0.1 mL in the plantar surface of the right hind paw.[15,34] Behavioral experiments and tissue collection were conducted 48 hours after the injection of CFA. The 48-hour experimental time point is common across many studies of inflammatory pain, including our previous work.[1,16] Forty-eight hours is both sufficient for the development of sensory hypersensitivity and edema, as well as the earliest time point at which sensory hypersensitivity stabilizes and becomes persistent.

2.2. Western blot analysis

Subcellular fractionation to obtain the synaptosomal fraction was performed as previously described.[5,6,31] In brief, PAG from both hemispheres of individual rats were homogenized in 0.32 M sucrose solution containing 0.1 mM $CaCl_2$, protease, and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). The homogenate was then adjusted to a final concentration of 1.25 M sucrose by adding 2 M sucrose and 0.1 mM $CaCl_2$. The homogenate was placed in an ultracentrifuge tube, overlaid with 1 M sucrose, and centrifuged at 100,000*g* for 3 hours at 4°C. The synaptosomal fraction was collected at the 1.25 m/1 m sucrose interface and then solubilized in 20 mm Tris-HCl, pH 6.0, buffer containing 1% Triton X-100 (TX-100) for 20 minutes at 4°C with gentle rotation.

Equal amounts of protein (10 μg) were loaded and separated in 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. We used a c-terminal CB1 antibody, (Dr. Ken Mackie, Indiana University), which recognizes a band at 52 kDa that does not appear in knock out animals.[9] Membranes were incubated overnight at 4°C with the CB1 antibody (1:1000). The membranes were then incubated with HRP-conjugated secondary antibody. After extensive washes, blots were visualized by enhanced chemiluminescence (ECLplus; GE Healthcare) according to the manufacturer's instructions. To ensure equal protein loading, membranes were reprobed with an antibody selective to actin (Millipore Bioscience Research Reagents). Actin was chosen as a loading control.[29] Actin levels did not change across any

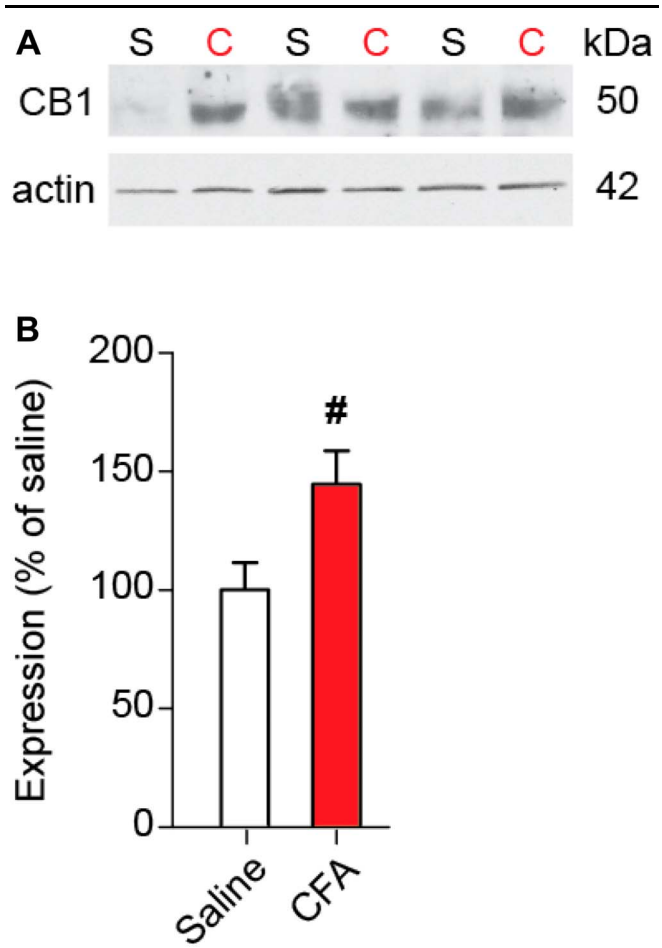

Figure 1. Western blot analyses of CB1 receptor expression in synaptosomal fractions from the PAG of male rats. (A) Relative to saline-injected rats (N = 9, black S), protein levels of the CB1 receptor were enhanced in animals injected with CFA in the hind paw (N = 9, red C). (B) Quantification of protein analysis in A, normalized to actin and saline-injected animals. #$P < 0.05$, unpaired 2-tailed *t* test. CFA, Complete Freund Adjuvant; PAG, periaqueductal gray.

Table 1

Primer sequences for real-time RT PCR of control and target genes.

Primer name	Primer sequence	Reference
Actin	Forward primer: 5′-TCTGTGTGGATTGGTGGCTCTA-3′ Reverse primer: 5′-CTGCTTGCTGATCCACATCTG-3′	Jin et al., 2014[18]
CB1	Forward primer: 5′-CCATTTCAAGCAAGGAGCAC-3′ Reverse primer: 5′-GTCATTCGAGCCCACGTAGA-3′	Jin et al., 2014

RT-PCR, reverse transcription polymerase chain reaction.

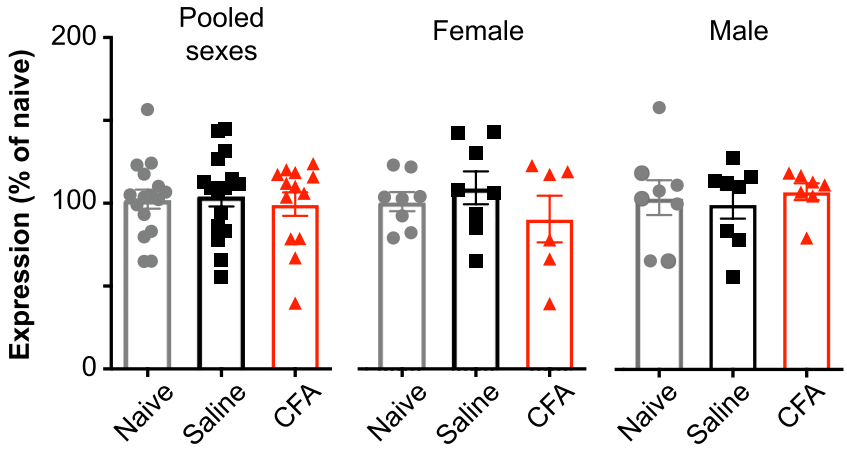

Figure 2. Quantitative polymerase chain reaction analyses of CB1 mRNA in the PAG of male and female rats with/without persistent inflammatory pain. CB1 mRNA levels in noninjected naïve controls (N = 16, gray symbols) was similar to that from male and female rats injected in the hind paw with either saline (N = 16, black symbols) or CFA (N = 13, red symbols, $F(2,42) = 0.16$, $P = 0.86$). mRNA levels normalized to naïve rats, data in the left panel are a composite of center and right panels. CFA, Complete Freund Adjuvant; PAG, periaqueductal gray.

pain treatment for the experiments. Quantification was performed using densitometry measurements in ImageJ, by comparing the intensity of the band with protein-specific antibodies to the actin band intensity to control for variation in loading and transfer. These values were normalized to saline-injected control values.

2.3. RNA extraction and quantitative real-time reverse transcription polymerase chain reaction

Immediately after sacrifice, brains were rapidly removed, bilateral PAG dissected, and stored in −80°C until further processing. Total PAG RNA was extracted and purified using RNeasy Mini Kit (Qiagen, Germantown, MD), following the manufacturer's instructions. The concentration and purity of RNA samples were detected by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). RNA was reverse-transcribed using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories). Reactions were incubated in a T100 Thermal Cycler (Bio-Rad Laboratories) initially at 25°C for 5 minutes, then at 46°C for 20 minutes, 95°C for 1 minute, and hold at 4°C. cDNA was amplified with specific primers (**Table 1**), using Power Up SYBR Green Master Mix (Applied Biosystems, Foster City, CA) on the QuantStudio 6 Flex Real-Time PCR System. The real-rime reverse transcription polymerase chain reaction process was first held at 50°C for 2 minutes, then at 95°C for 10 minutes, followed by 40 cycles of thermal cycling at 95°C for 15 seconds, and 57°C for 1 minute, finally the melt curve stage was initiated with 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. The fold change in gene expression was calculated using $2^{-\Delta\Delta Ct}$ method (**Table 1**) with actin as the internal control. The presented data were normalized to the pain-naïve group.

2.4. Mixed CB1/CB2 agonist-stimulated GTPγS autoradiography

Brains were collected from saline-injected and CFA-injected rats 48 hours after intrapaw injection. Brains were snap frozen with isopentane at −30°C and stored at −80°C until further processing. On the day of processing, brains were sectioned coronally using a cryostat (16-μm-thick sections) at −20°C. Sections were thaw mounted on Superfrost charged slides. Sections were preincubated in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, 2 mM GDP, 1 μM DPCPX, and pH 7.4) for 15 minutes. Agonist-stimulated CB1 activity was determined by incubating brain sections in [^{35}S]GTPγS (40 PM) with vehicle or the pan-cannabinoid agonist CP55,940 (10 μM) for 2 hours at RT. After incubation, slides were washed 2 times in ice-cold wash buffer (50 mm Tris-HCl, pH 7.4) followed by a brief wash in ice-cold deionized water (30 seconds). Slides were air dried at room temperature for 30 minutes before exposure to a phosphor-imaging plate (GE BAS-IP SR 2025 E, high resolution) together with [^{14}C] standards for 2 d, before scanning with a Typhoon 9410 variable mode imager (GE Healthcare). Data were normalized to a [^{14}C] standard curve in uCi/g within NIH Fiji ImageJ software. Data from the resulting agonist-stimulated samples were compared with non–agonist-treated brain samples to determine the percentage activation of CB1 above basal.

2.5. Statistics

Data are presented as mean ± SEM. The statistical significance was assessed using either unpaired *t* test, or 1-way analysis of variance. Significance was assessed at $P < 0.05$. Statistical analyses were performed using GraphPad Prism 8.

During analysis of the GTPγS data, we conducted an effect size analysis using the variance from the observed results, allowing us to determine if the study was sufficiently powered for the female data sets. The female data revealed the actual power was 95.13% and that it would require an N = 33 per group to reveal potential differences. Based on these analyses, we concluded that the GTPγS experiments were sufficiently powered.

3. Results

Previous studies in male rats have demonstrated that persistent pain upregulates the expression of the CB1 receptor in pain-related regions such as the thalamus and dorsal horn.[23,36,43] We first sought to assess whether similar inflammatory pain–induced upregulation occurs in the PAG. We dissected PAG tissue 48 hours after male rats were injected in the hind paw with either saline or CFA. Because of the role of the CB1 receptor in

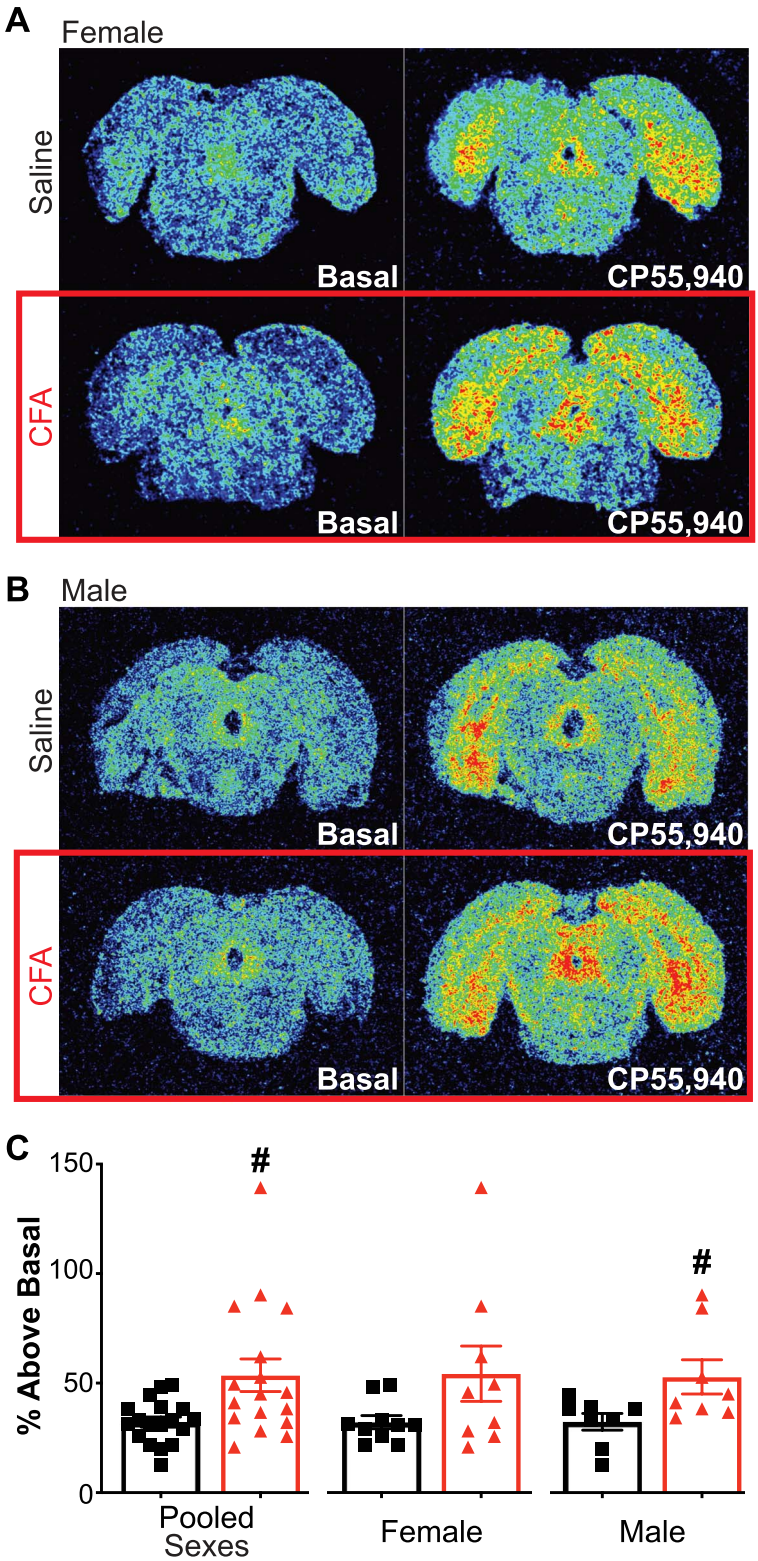

Figure 3. Cannabinoid receptor agonist-stimulated G-protein coupling in the PAG of rats with/without persistent inflammatory pain. (A) Representative slice autoradiograph from female rats with hind paw injections of either saline (top panels, N = 9) or CFA (bottom panels, N = 10). G-protein activity was measured before (left panels) and after incubation with the pan-cannabinoid agonist CP55,940. (B) Representative slice autoradiograph from male rats with hind paw injections of either saline (top panels, N = 8) or CFA (bottom panels, N = 8). (C) Quantification of agonist-stimulated G-protein activation in the PAG from animals injected with saline (black symbols) or CFA (red symbols), relative to the basal activity. Data in the left panel are a composite of center and right panels, # indicates $P < 0.05$, unpaired 2-tailed *t* test. CFA, Complete Freund Adjuvant; PAG, periaqueductal gray.

retrograde signaling and synaptic release in the PAG,[40] we used a synaptosomal fraction protocol to focus on synaptically expressed CB1 receptor proteins.[5,31] Western blot analyses from synaptosomal fractions revealed that compared with saline-treated animals, CB1 receptor expression was enhanced in CFA-injected animals (48 hours after CFA injection, $t(16) = 2.45$, $P = 0.03$, **Fig. 1**).

To determine whether pain-induced increases in CB1 protein expression were accompanied by an increase in CB1 mRNA, we conducted qPCR analysis. Because previous studies have demonstrated sex differences in inflammatory pain–induced upregulation of CB1 mRNA, we expanded our subsequent studies to include both male and female rats.[32] Forty-eight hours after CFA injection in the hind paw, levels of CB1 mRNA were similar in PAG tissue from naïve (noninjected), saline-injected, and CFA-injected rats (**Fig. 2**). There were also no sex differences in CB1 mRNA expression. Because CB1 protein expression was not accompanied by an enhancement in mRNA expression, we hypothesize that the presence of inflammatory pain recruits internal CB1 receptors to the membrane of neurons within the PAG or alternatively enhances the coupling of CB1 receptors to effector signaling cascades.

An increase in synaptosomal CB1 receptor expression could result in an increase in the ability of cannabinoids to modulate pain. Therefore, we next evaluated functional adaptations in CB1 receptor signaling in male and female rats. To test this hypothesis, we assessed whether CFA enhances the ability of CB1 agonists to activate G-protein coupling, using radiolabelled nonhydrolysable [^{35}S]-GTPγS (**Fig. 3A and B**). Compared with the basal G-protein activity, the pan-cannabinoid agonist CP55,940 (10 uM) induced greater bilateral G-protein coupling in PAG slices from CFA-treated rats than saline-treated (left panel **Fig. 3C**, $t(33) = 2.82$, $P = 0.01$). This effect seems to be driven by the significant pain-induced enhancement of G-protein coupling in male animals (right panel, **Fig. 3C**, $t(14) = 2.35$, $P = 0.03$). In females, CFA treatment induced a trend toward enhanced G-protein coupling in the PAG (**Fig. 3C** center panel), although this effect failed to reach statistical significance.

4. Discussion

This work revealed that persistent inflammatory pain causes an upregulation in CB1 receptor expression in synaptosomal fractions from the PAG in rats. Because this enhanced protein expression is not accompanied by increases in CB1 mRNA, it is likely that inflammatory pain promotes insertion of extant CB1 proteins into neuronal membranes in the PAG. Alternatively, CB1 upregulation may be occurring on afferent projections from other brain regions, given the pre-synaptic distribution of the CB1 receptor. This could account for our observed difference between CB1 protein and mRNA expression within the PAG. Although our previous ultrastructural studies have demonstrated that there is indeed a large intracellular population of CB1 proteins in the PAG under basal conditions,[45] the mechanisms by which pain may recruit these receptors to the membrane are not well characterized. However, chronic cannabinoid exposure and constitutive activity at the CB1 receptor lead to desensitization, downregulation, and recycling of CB1 receptors.[20,38] Because of the critical role of the endocannabinoid system in nociceptive signaling,[46] it is perhaps unsurprising that persistent inflammatory pain may enhance endogenous cannabinoid tone, resulting in constitutive activity at CB1 receptors and alterations in their synaptosomal expression in the PAG.

The enhanced expression of CB1 proteins is accompanied by an enhanced ability for cannabinoid agonists to couple with G-protein signaling cascades in the PAG. This pain-induced enhancement of G-protein signaling could be sex-specific, with males exhibiting a greater enhancement in pain-induced G-protein activation. A large body of research has shown sex differences in the functional connectivity of the PAG, the analgesic properties of cannabinoids, and opioid modulation of pain.[1,4,10,18,25,41] Sexually dimorphic pain-induced changes in endocannabinoid tone could contribute to what appears to be a sex-specific phenomenon. Here, we have observed that CP55,940-stimulated G-protein coupling is enhanced by the presence of pain in males, but merely trends toward enhancement in females. However, the presence of pain itself may enhance tonic endogenous cannabinoid signaling in females, which could result in a ceiling effect of CP55,940 stimulation. That is, basal endogenous tone could be occluding the effect of agonist stimulation in females, manifesting as a mild CP55,940 effect, as we observe here. Endogenous cannabinoid tone is highly dynamic, and similar results (female insensitivity to cannabinoid agonists and enhanced endocannabinoid tone) have been observed in other experimental paradigms,[14,30,44] including CFA-induced enhancement of endocannabinoid tone in the descending pain pathway.[21]

The CB2 receptor may also play a role in this observed sex difference. Because CP55,940 is a pan-cannabinoid agonist, some of our observed pain-enhanced G-protein activation in the GTPγS assay may be due to sexually dimorphic pain-induced alterations in the CB2 receptor. Similar to what is observed in neuropathic pain[47,48] and morphine administration,[24] CFA may induce an upregulation in the expression and/or function of CB2 receptors in the PAG. This is quite plausible, given that CFA enhances the function of CB2 receptors in the dorsal root ganglion, as well as rostral ventromedial medulla (RVM), which is the downstream target of the PAG in the descending pain pathway.[17,21] Other stress-inducing experimental procedures such as repeated tail-shock, maternal separation, and chronic drug exposure also lead to sexually dimorphic alterations in CB2 function.[27,37,49]

Dysregulation of the endogenous cannabinoid system has been implicated in several chronic diseases that disproportionately affect women, including fibromyalgia and migraine.[35] Our results support this notion and indicate that both endogenous cannabinoid tone and cannabinoid receptor signaling are impacted by the presence of persistent pain states. Novel pharmacotherapies which target the endogenous cannabinoid system may be a particularly useful strategy for coping with chronic pain, particularly for women.

Disclosures

The authors have no conflicts of interest to declare.

Acknowledgements

This work was funded by NIDA K99/R00 DA041467 (A.W.P.), DA045463 (J.A.M.), DA041781 (J.A.M.), and DA 005010 (C.M.C.) and Shirley and Stefan Hatos Foundation (C.M.C.).

Article history:
Received 16 October 2020
Received in revised form 10 December 2020

Accepted 25 December 2020
Available online 5 March 2021

References

[1] Averitt DL, Eidson LN, Doyle HH, Murphy AZ. Neuronal and glial factors contributing to sex differences in opioid modulation of pain. Neuropsychopharmacology 2019;44:155–65.
[2] Bair MJ, Robinson RL, Katon W, Kroenke K. Depression and pain comorbidity: a literature review. Arch Intern Med 2003;163:2433–45.
[3] Bajic D, Monory K, Conrad A, Maul C, Schmid RM, Wotjak CT, Stein-Thoeringer CK. Cannabinoid receptor type 1 in the brain regulates the affective component of visceral pain in mice. Neuroscience 2018;384: 397–405.
[4] Bernal SA, Morgan MM, Craft RM. PAG mu opioid receptor activation underlies sex differences in morphine antinociception. Behav Brain Res 2007;177:126–33.
[5] Billa SK, Liu J, Bjorklund NL, Sinha N, Fu Y, Shinnick-Gallagher P, Moron JA. Increased insertion of glutamate receptor 2-lacking alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors at hippocampal synapses upon repeated morphine administration. Mol Pharmacol 2010;77:874–83.
[6] Billa SK, Sinha N, Rudrabhatla SR, Moron JA. Extinction of morphine-dependent conditioned behavior is associated with increased phosphorylation of the GluR1 subunit of AMPA receptors at hippocampal synapses. Eur J Neurosci 2009;29:55–64.
[7] Cahill CM, Xue L, Grenier P, Magnussen C, Lecour S, Olmstead MC. Changes in morphine reward in a model of neuropathic pain. Behav Pharmacol 2013;24:207–13.
[8] Descalzi G, Mitsi V, Purushothaman I, Gaspari S, Avrampou K, Loh YE, Shen L, Zachariou V. Neuropathic pain promotes adaptive changes in gene expression in brain networks involved in stress and depression. Sci Signal 2017;10:eaaj1549.
[9] Deshmukh S, Onozuka K, Bender KJ, Bender VA, Lutz B, Mackie K, Feldman DE. Postnatal development of cannabinoid receptor type 1 expression in rodent somatosensory cortex. Neuroscience 2007;145: 279–87.
[10] Doyle HH, Murphy AZ. Sex-dependent influences of morphine and its metabolites on pain sensitivity in the rat. Physiol Behav 2018;187:32–41.
[11] Finn DP, Jhaveri MD, Beckett SR, Roe CH, Kendall DA, Marsden CA, Chapman V. Effects of direct periaqueductal grey administration of a cannabinoid receptor agonist on nociceptive and aversive responses in rats. Neuropharmacology 2003;45:594–604.
[12] Franklin TB, Silva BA, Perova Z, Marrone L, Masferrer ME, Zhan Y, Kaplan A, Greetham L, Verrechia V, Halman A, Pagella S, Vyssotski AL, Illarionova A, Grinevich V, Branco T, Gross CT. Prefrontal cortical control of a brainstem social behavior circuit. Nat Neurosci 2017;20: 260–70.
[13] Grenier P, Mailhiot MC, Cahill CM, Olmstead MC. Blockade of dopamine D1 receptors in male rats disrupts morphine reward in pain naive but not in chronic pain states. J Neurosci Res 2019. doi: 10.1002/jnr.24553 [Epub ahead of print].
[14] Henricks AM, Berger AL, Lugo JM, Baxter-Potter LN, Bieniasz KV, Petrie G, Sticht MA, Hill MN, McLaughlin RJ. Sex- and hormone-dependent alterations in alcohol withdrawal-induced anxiety and corticolimbic endocannabinoid signaling. Neuropharmacology 2017;124:121–33.
[15] Hipolito L, Wilson-Poe A, Campos-Jurado Y, Zhong E, Gonzalez-Romero J, Virag L, Whittington R, Comer SD, Carlton SM, Walker BM, Bruchas MR, Moron JA. Inflammatory pain promotes increased opioid self-administration: role of dysregulated ventral tegmental area mu opioid receptors. J Neurosci 2015;35:12217–31.
[16] Ho YC, Lin TB, Hsieh MC, Lai CY, Chou D, Chau YP, Chen GD, Peng HY. Periaqueductal gray glutamatergic transmission governs chronic stress-induced depression. Neuropsychopharmacology 2018;43:302–12.
[17] Hsieh GC, Pai M, Chandran P, Hooker BA, Zhu CZ, Salyers AK, Wensink EJ, Zhan C, Carroll WA, Dart MJ, Yao BB, Honore P, Meyer MD. Central and peripheral sites of action for CB(2) receptor mediated analgesic activity in chronic inflammatory and neuropathic pain models in rats. Br J Pharmacol 2011;162:428–40.
[18] LaFleur RA, Wilson RP, Morgan DJ, Henderson-Redmond AN. Sex differences in antinociceptive response to Delta-9-tetrahydrocannabinol and CP 55,940 in the mouse formalin test. Neuroreport 2018;29:447–52.
[19] Landgraf D, Long JE, Welsh DK. Depression-like behaviour in mice is associated with disrupted circadian rhythms in nucleus accumbens and periaqueductal grey. Eur J Neurosci 2016;43:1309–20.
[20] Leterrier C, Laine J, Darmon M, Boudin H, Rossier J, Lenkei Z. Constitutive activation drives compartment-selective endocytosis and axonal targeting of type 1 cannabinoid receptors. J Neurosci 2006;26:3141–53.
[21] Li MH, Suchland KL, Ingram SL. Compensatory activation of cannabinoid CB2 receptor inhibition of GABA release in the rostral ventromedial medulla in inflammatory pain. J Neurosci 2017;37:626–36.
[22] Lichtman AH, Cook SA, Martin BR. Investigation of brain sites mediating cannabinoid-induced antinociception in rats: evidence supporting periaqueductal gray involvement. J Pharmacol Exp Ther 1996;276: 585–93.
[23] Lim G, Sung B, Ji RR, Mao J. Upregulation of spinal cannabinoid-1-receptors following nerve injury enhances the effects of Win 55,212-2 on neuropathic pain behaviors in rats. PAIN 2003;105:275–83.
[24] Lim G, Wang S, Mao J. Central glucocorticoid receptors modulate the expression of spinal cannabinoid receptors induced by chronic morphine exposure. Brain Res 2005;1059:20–7.
[25] Linnman C, Beucke JC, Jensen KB, Gollub RL, Kong J. Sex similarities and differences in pain-related periaqueductal gray connectivity. PAIN 2012;153:444–54.
[26] Liu SS, Pickens S, Burma NE, Ibarra-Lecue I, Yang H, Xue L, Cook C, Hakimian JK, Severino AL, Lueptow L, Komarek K, Taylor AMW, Olmstead MC, Carroll FI, Bass CE, Andrews AM, Walwyn W, Trang T, Evans CJ, Leslie FM, Cahill CM. Kappa opioid receptors drive a tonic aversive component of chronic pain. J Neurosci 2019;39:4162–78.
[27] Llorente-Berzal A, Assis MA, Rubino T, Zamberletti E, Marco EM, Parolaro D, Ambrosio E, Viveros MP. Sex-dependent changes in brain CB1R expression and functionality and immune CB2R expression as a consequence of maternal deprivation and adolescent cocaine exposure. Pharmacol Res 2013;74:23–33.
[28] Massaly N, Copits BA, Wilson-Poe AR, Hipolito L, Markovic T, Yoon HJ, Liu S, Walicki MC, Bhatti DL, Sirohi S, Klaas A, Walker BM, Neve R, Cahill CM, Shoghi KI, Gereau RWt, McCall JG, Al-Hasani R, Bruchas MR, Moron JA. Pain-induced negative affect is mediated via recruitment of the nucleus accumbens kappa opioid system. Neuron 2019;102:564–73 e566.
[29] Matus A, Ackermann M, Pehling G, Byers HR, Fujiwara K. High actin concentrations in brain dendritic spines and postsynaptic densities. Proc Natl Acad Sci U S A 1982;79:7590–4.
[30] Melis M, De Felice M, Lecca S, Fattore L, Pistis M. Sex-specific tonic 2-arachidonoylglycerol signaling at inhibitory inputs onto dopamine neurons of Lister Hooded rats. Front Integr Neurosci 2013;7:93.
[31] Moron JA, Abul-Husn NS, Rozenfeld R, Dolios G, Wang R, Devi LA. Morphine administration alters the profile of hippocampal postsynaptic density-associated proteins: a proteomics study focusing on endocytic proteins. Mol Cell Proteomics 2007;6:29–42.
[32] Niu KY, Zhang Y, Ro JY. Effects of gonadal hormones on the peripheral cannabinoid receptor 1 (CB1R) system under a myositis condition in rats. PAIN 2012;153:2283–91.
[33] Okine BN, Madasu MK, McGowan F, Prendergast C, Gaspar JC, Harhen B, Roche M, Finn DP. N-palmitoylethanolamide in the anterior cingulate cortex attenuates inflammatory pain behaviour indirectly via a CB1 receptor-mediated mechanism. PAIN 2016;157:2687–96.
[34] Parent AJ, Beaudet N, Beaudry H, Bergeron J, Berube P, Drolet G, Sarret P, Gendron L. Increased anxiety-like behaviors in rats experiencing chronic inflammatory pain. Behav Brain Res 2012;229:160–7.
[35] Russo EB. Clinical endocannabinoid deficiency reconsidered: current research supports the theory in migraine, fibromyalgia, irritable bowel, and other treatment-resistant syndromes. Cannabis Cannabinoid Res 2016;1:154–65.
[36] Siegling A, Hofmann HA, Denzer D, Mauler F, De Vry J. Cannabinoid CB(1) receptor upregulation in a rat model of chronic neuropathic pain. Eur J Pharmacol 2001;415:R5–7.
[37] Suarez J, Llorente R, Romero-Zerbo SY, Mateos B, Bermudez-Silva FJ, de Fonseca FR, Viveros MP. Early maternal deprivation induces gender-dependent changes on the expression of hippocampal CB(1) and CB(2) cannabinoid receptors of neonatal rats. Hippocampus 2009;19:623–32.
[38] Thibault K, Carrel D, Bonnard D, Gallatz K, Simon A, Biard M, Pezet S, Palkovits M, Lenkei Z. Activation-dependent subcellular distribution patterns of CB1 cannabinoid receptors in the rat forebrain. Cereb Cortex 2013;23:2581–91.
[39] Tortorici V, Robbins CS, Morgan MM. Tolerance to the antinociceptive effect of morphine microinjections into the ventral but not lateral-dorsal periaqueductal gray of the rat. Behav Neurosci 1999;113:833–9.
[40] Vaughan CW, Connor M, Bagley EE, Christie MJ. Actions of cannabinoids on membrane properties and synaptic transmission in rat periaqueductal gray neurons in vitro. Mol Pharmacol 2000;57:288–95.
[41] Wakley AA, Wiley JL, Craft RM. Gonadal hormones do not alter the development of antinociceptive tolerance to delta-9-

tetrahydrocannabinol in adult rats. Pharmacol Biochem Behav 2015;133: 111–21.
[42] Wang P, Li H, Barde S, Zhang MD, Sun J, Wang T, Zhang P, Luo H, Wang Y, Yang Y, Wang C, Svenningsson P, Theodorsson E, Hokfelt TG, Xu ZQ. Depression-like behavior in rat: involvement of galanin receptor subtype 1 in the ventral periaqueductal gray. Proc Natl Acad Sci U S A 2016;113: E4726–4735.
[43] Wang S, Lim G, Mao J, Sung B, Yang L, Mao J. Central glucocorticoid receptors regulate the upregulation of spinal cannabinoid-1 receptors after peripheral nerve injury in rats. Pain 2007;131:96–105.
[44] Wiley JL, Jones AR, Wright MJ Jr. Exposure to a high-fat diet decreases sensitivity to Delta9-tetrahydrocannabinol-induced motor effects in female rats. Neuropharmacology 2011;60:274–83.
[45] Wilson-Poe AR, Morgan MM, Aicher SA, Hegarty DM. Distribution of CB1 cannabinoid receptors and their relationship with mu-opioid receptors in the rat periaqueductal gray. Neuroscience 2012;213:191–200.
[46] Woodhams SG, Sagar DR, Burston JJ, Chapman V. The role of the endocannabinoid system in pain. Handb Exp Pharmacol 2015;227: 119–43.
[47] Wotherspoon G, Fox A, McIntyre P, Colley S, Bevan S, Winter J. Peripheral nerve injury induces cannabinoid receptor 2 protein expression in rat sensory neurons. Neuroscience 2005;135:235–45.
[48] Wu J, Hocevar M, Bie B, Foss JF, Naguib M. Cannabinoid type 2 receptor system modulates paclitaxel-induced microglial dysregulation and central sensitization in rats. J Pain 2019;20:501–14.
[49] Xing G, Carlton J, Jiang X, Wen J, Jia M, Li H. Differential expression of brain cannabinoid receptors between repeatedly stressed males and females may play a role in age and gender-related difference in traumatic brain injury: implications from animal studies. Front Neurol 2014;5:161.
[50] Zhu H, Xiang HC, Li HP, Lin LX, Hu XF, Zhang H, Meng WY, Liu L, Chen C, Shu Y, Zhang RY, Zhang P, Si JQ, Li M. Inhibition of GABAergic neurons and excitation of glutamatergic neurons in the ventrolateral periaqueductal gray participate in electroacupuncture analgesia mediated by cannabinoid receptor. Front Neurosci 2019;13:484.